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Efficacy of Azole Derivatives, Clotrimazole, Itraconazole, and Voriconazole on Cystic Echinococcosis protoscolices: A Comparative Study

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Abstract

Cystic echinococcosis (CE) presents significant public health challenges in various regions worldwide. The adverse effects and limited efficacy of current scolicidal agents, underscore the urgent need for novel therapeutics developments. This study aimed to evaluate the scolicidal effects of the azole compounds clotrimazole, itraconazole, and voriconazole on CE protoscolices. Protoscolices were aseptically harvested from ovine hepatic hydatid cysts. After confirming high viability (over 90%) through eosin staining, the protoscolices were subjected to graded concentrations of clotrimazole, itraconazole, and voriconazole. A 20% saline solution served as the positive control, while a DMSO solution (the drug solvent) used as the negative control. Following a three-hour exposure period, eosin staining and microscopic examination were employed to determine the proportion of viable protoscolices. Statistical analysis was conducted using SPSS software and ANOVA tests. The results showed that the percentage of dead protoscolices in the presence of clotrimazole was significantly higher compared to the negative control and both itraconazole and voriconazole, although it was significantly lower than the positive control. The highest mortality rate in the clotrimazole group was observed at 1024 µg/mL, corresponding to an 89.9±63.69% mortality rate. The data also indicated a statistically significant correlation between increasing clotrimazole concentration and its mortality rate (p=0.011). These findings suggest that clotrimazole exhibits superior scolicidal activity against CE protoscolices compared to itraconazole and voriconazole, indicating its potential as an anti-parasitic medication, suitable for alternative or adjunctive treatment of this debilitating disease.

Keywords: Hydatid cyst, protoscolices, azoles, clotrimazole, itraconazole, voriconazole.

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1. Introduction

Cystic echinococcosis (CE), otherwise known as hydatid cyst, is a zoonotic parasitic disease caused by the larval stage of the *Echinococcus granulosus* tapeworm (1, 2). The disease is prevalent in many parts of the world, particularly in rural areas where there is close contact between humans and infected animals (3-5). The treatment of CE involves a combination of surgical intervention and pharmacological therapy. Surgery is often necessary to remove cysts from vital organs such as the liver or lungs. However, pharmacological therapy plays a crucial role in preventing re-

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currence and managing cases (2, 6).

Azole derivatives are a class of antifungal drugs that inhibit the synthesis of ergosterol, an essential component of fungal cell membranes (7). By disrupting membrane integrity, azole derivatives exert their antifungal activity. Azole derivatives have shown promising effects on protoscolices, which are the germinal cells found within hydatid cysts (6, 8-10).

In recent years, studies have explored the potential use of azole derivatives as an alternative or adjunctive treatment for echinococcosis (11). Several azole drugs, including albendazole and mebendazole, have been investigated for their efficacy against protoscolices both *in vitro* and *in vivo* (12-15). *In vitro* studies have demonstrated that azole derivatives can effectively kill protoscolices at various concentrations. They have been shown to induce morphological changes such as vacuolation and degeneration in protoscolices' tegumental layers. Furthermore, these drugs have been found to inhibit protoscolex viability by interfering with their metabolic processes (12, 13).

In vivo studies using animal models have provided evidence supporting the efficacy of azole derivatives against *Echinococcus* larvae. These studies have shown that treatment with azole drugs can significantly reduce cyst size and viability in infected animals, corroborating the potential of these compounds as effective therapeutic agents in managing echinococcosis (8, 9, 13).

Despite these promising findings, there are still challenges associated with using azole derivatives for treating echinococcosis. One major limitation is their poor bioavailability due to low solubility and extensive first-pass metabolism (6, 7). This necessitates high doses or prolonged treatment durations to achieve therapeutic concentrations at target sites.

One of the main azole derivatives that are currently used in the treatment of hydatid cysts is albendazole. Albendazole is a broad-spectrum anthelmintic drug that is considered a cornerstone in the medical management of hydatid cysts, either as a standalone treatment or in combination with surgical or percutaneous interventions (6, 16).

Albendazole's mechanism of action involves inhibiting the polymerization of the parasite's tubulin into microtubules, thereby disrupting essential cellular processes such as nutrient uptake, metabolic processes, and the parasite's structural integrity. Consequently, this leads to the degeneration of the hydatid cyst (6). Albendazole has been shown to be effective in inactivating the germinal layer of the hydatid cyst, preventing the production of new daughter cysts, and reducing the size of the cysts over time. It is also used as a prophylactic measure to prevent secondary hydatidosis caused by spillage of cyst contents during surgical interventions. However, the effectiveness of albendazole can be variable, with some cysts showing resistance (2, 17). This may be due to factors such as the stage of the cyst, the strain of the parasite, and the duration of treatment. In the present study, we evaluated the protoscolicidal effect of three azole derivatives including clotrimazole, itraconazole, and voriconazole in an in-vitro environment.

2. Material and Methods

2.1. Preparation of different concentrations of compounds

The study utilized three azole derivatives, Itraconazole, Clotrimazole, and Voriconazole, purchased from Sigma Aldrich. These azole compounds were dissolved in DMSO solvent, and serial dilutions were prepared in accordance with the CLSI protocol, ranging from 64 to 1024 µg/mL. The selected concentrations were based on preliminary study findings.

2.2. Preparation of culture medium containing protoscoleces

Fresh, fertile cysts containing protoscoleces were collected from sheep livers in coordination with Shiraz industrial slaughterhouse and transferred to Parasitology Laboratory at Shiraz University of Medical Sciences. Under sterile conditions, protoscolices were aspirated from hepatic cysts and rinsed with sterile PBS buffer. They were then maintained in RPMI 1640 culture medium supplemented

with 10% fetal bovine serum (FBS) at 37 °C. The viability of protoscolices was assessed using eosin vital staining, and only those with a viability exceeding 90% were included in the study.

2.3. Exposure of protoscoleces to drugs and evaluation of compound's efficacy

Protoscolices were exposed to serial concentrations of the drugs. After a three-hour exposure period, eosin vital staining was employed to evaluate the proportion of drug-induced protoscolex mortality. Under light microscopy, live protoscolices, which are impermeable to red eosin dye, were distinguishable from dead protoscolices, which absorbed the dye and appeared red. The efficacy of these compounds was evaluated in conjunction with a compound routinely used in surgical practice, 20% normal saline, as a positive control. Additionally, the drug solvent served as a negative control.

2.4. Statistical analysis

Statistical analysis of the collected data was performed using SPSS software (version 20). ANOVA was used to compare the mean percentage of dead protoscoleces in different groups under investigation.

3. Results

The mean and standard error of the percentage of protoscolex mortality after three-hour exposure to graded concentrations of the investigated drugs, as well as the positive and negative controls, are presented in Tables 1 and Figure 1.

The mean of protoscolex mortality rate across varying concentrations of clotrimazole was significantly higher than that of itraconazole. Similarly, clotrimazole demonstrated a significantly higher mortality rate compared to voriconazole. While itraconazole exhibited a higher mortality rate than voriconazole, this difference was not statistically significant.

The mean percentage of protoscolex mortality in the presence of different concentrations of clotrimazole was significantly higher compared to the negative control group, but lower than the positive control group. The highest mortality rate in the clotrimazole group was observed at a concentration of 1024 µg/mL, corresponding to an 89.9±63.69% mortality rate. The data also indicated a statistically significant correlation between increasing clotrimazole concentration and its mortality rate (P=0.011).

The mortality rate of protoscoleces exposed to itraconazole was significantly lower compared to the positive control group. However, the difference in mortality rate between the itraconazole and negative control groups was statistically significant. The highest mortality rate in the itraconazole group was observed at a concentration of 1024 µg/mL, equivalent to an 89.9±80.31%. mortality rate The effect of increasing itraconazole concentration on its mortality rate was not statistically significant (P=0.99). The average percentage of protoscolex mortality in the presence

Table 1. Mortality rate (percent) of protoscolices at different concentrations of azole derivatives.

	64 µg/mL	128 µg/mL	256 µg/mL	512 µg/mL	1024 µg/mL
Clotrimazole	46.95±9.89	50.68±9.89	64.61±9.89	65.16±9.89	69.69±9.89
Itraconazole	9.14±9.89	12.86±9.89	29.79±9.89	27.33±9.89	31.80±9.89
Voriconazole	6.19±9.89	9.91±9.89	23.84±9.89	24.39±9.89	28.86±9.89
Positive control (20% saline)			85.52±14.48		
Negative control (drug solvent)			13.26±14.48		

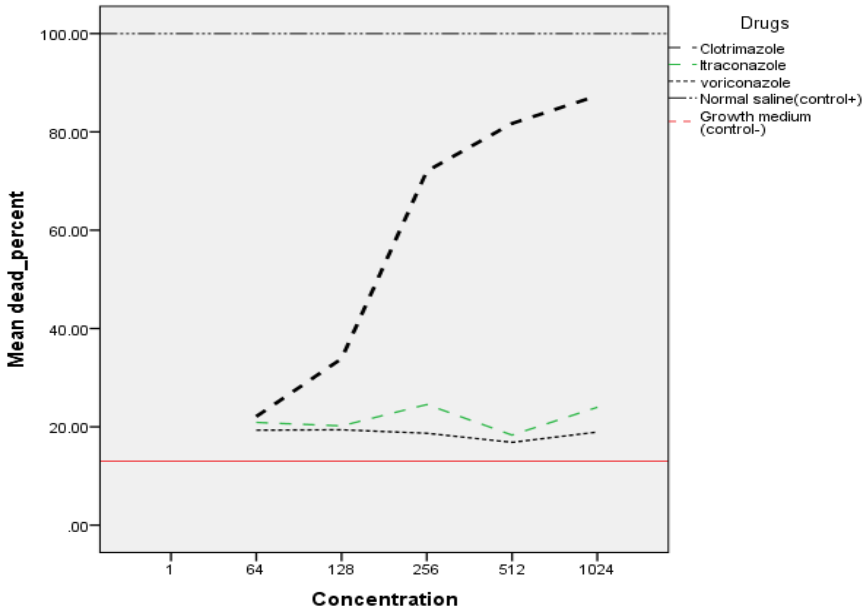

Figure 1. The effect of different concentrations of azole derivatives on protoscolices of cystic echinococcosis.

of varying concentrations of voriconazole was lower compared to the positive control group and higher than the negative control group, but this difference was not statistically significant. The highest mortality rate in the voriconazole group was observed at a concentration of 1024 µg/mL, equivalent to an 89.9±86.28% mortality rate. The effect of increasing voriconazole concentration on its mortality rate was not statistically significant (P=1.00).

4. Discussion

Cystic echinococcosis is a significant zoonotic disease prevalent in several countries around the world with significant commercial and health impacts (1, 3, 17, 18). The primary and most effective treatment modalities for CE include surgical cyst excision or Puncture Aspiration Injection Re-aspiration (PAIR), wherein scolicidal agents are employed to prevent recurrence and mitigate side effects (2). Historically, various scolicidal solutions, including, formalin and hydrogen peroxide, have been utilized, but their use has been discontinued due to severe adverse effects. Ethyl alcohol (95%) and saline (20%) also possess scolicidal properties, but their use has been associated with hepatic damage. These challenges highlight the urgent need for effective and safe scolicidal compounds for use during sur-

Table 2. The difference in the mean percentage of dead protoscolex in different azole derivative groups

	Mean difference	Standard error	P value
Clotrimazole/positive control	-40.56	15.89	0.045
Clotrimazole/negative control	46.12	15.89	0.017
Itraconazole/positive control	-78.14	15.89	0.001
Itraconazole/negative control	8.32	15.89	0.423
Voriconazole/positive control	-81.35	15.86	0.001
Voriconazole/negative control	5.37	15.89	0.604
Clotrimazole/Itraconazole	37.82	9.15	0.001
Clotrimazole/Voriconazole	4.77	9.15	0.001
Itraconazole/Voriconazole	2.94	9.15	0.749

gical procedures. On the other hand, the only drug used to treat this disease, i.e. albendazole, has, at most, an efficiency of about 60% (19). These challenges underscore the necessity to discover and develop new therapeutic agents for the treatment of this disease.

The present study investigated the scolicidal effects of three azole compounds, clotrimazole, itraconazole, and voriconazole, using an *in vitro* approach. Itraconazole and voriconazole demonstrated similar and modest scolicidal properties, with the average percentage of protoscolex mortality comparable to the negative control group. Clotrimazole exhibited a significantly higher protoscolex mortality rate than both voriconazole and itraconazole, albeit lower than the positive control (20% saline), with the difference being statistically significant. Azole derivatives have demonstrated promising effects on protoscolices of *Echinococcus* in various experimental settings (10, 20, 21). Their ability to disrupt membrane integrity and inhibit ergosterol synthesis contributes to their anti-parasitic activity against *Echinococcus* species. The superior anthelmintic properties of clotrimazole compared to other azoles may be attributed to its broader inhibitory spectrum on cytochrome P450 enzymes (22, 23).

A 2017 study by Mardanov *et al.* on the anthelmintic effects of azole compounds revealed variable anthelmintic activity, with the highest efficacy observed for miconazole and clotrimazole (24).

In 2018, Pakharukova *et al*. investigated the effects of combining praziquantel and clotrimazole on the tapeworm *Echinococcus granulosus*, finding that the combination enhanced the anthelmintic effects of clotrimazole (25).

Previous studies have primarily focused on the efficacy of benzimidazole derivatives, such as albendazole and its salts, against CE. These studies have shown that albendazole, particularly in its sulfoxide form, is effective in reducing the viability of protoscolices. However, the current study highlights that clotrimazole, an azole derivative, may offer a more potent alternative, especially at higher concentrations.

The molecular basis of azole derivatives' efficacy, including clotrimazole, involves disrupting the cell membrane integrity of the protoscolices, leading to increased mortality. This mechanism is consistent with the action of other azole derivatives, which inhibit ergosterol synthesis, a critical component of the cell membrane in fungi and some parasites.

While albendazole remains a standard treatment for CE, the current study suggests that clotrimazole could serve as a more effective scolicidal agent. This is particularly relevant in cases where resistance to benzimidazole derivatives is observed or when adjunctive therapy is required to enhance treatment efficacy.

The study's findings open avenues for further research into the clinical application of clotrimazole in treating CE. Its superior efficacy at higher concentrations warrants investigation into optimal dosing regimens and potential combination therapies with existing treatments like albendazole.

5. Conclusion

The identification of highly efficacious and minimally toxic scolicidal compounds is crucial for the management of CE. The findings of the present study showed that clotrimazole exhibited a greater protoscolicidal effect compared to the other two azole compounds, voriconazole and itraconazole. Although clotrimazole induced protoscolex death, given the duration of its exposure to protoscoleces and the percentage of protoscolex mortality compared to the positive control, it appears that this compound is not a suitable protoscolicidal compound for PAIR procedures. However, it can be considered as an anti-parasite medicine, similar to albendazole, with sufficient efficacy as an alternative or adjunctive treatment for this debilitating disease.

Conducting *in vivo* studies on this compound is essential to elucidate its efficacy and potential side effects in the management of hydatid cysts.

Also, future research should focus on elucidating the detailed mechanisms of action, optimizing dosing strategies, and evaluating the long-term outcomes of clotrimazole use in CE management.

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Authors contributions

Authors' Contribution: BS and KZ: conceived and designed the study, assisted with data analysis. and drafted the manuscript; and assisted with data analysis. MO, SAA, AP, and HM carried out the experiments. All authors read and approved the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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